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Heavy metal resistance and genotypic analysis of metal resistance genes in gram-positive and gram-negative bacteria present in Ni-rich serpentine soil and in the rhizosphere of *Alyssum murale*

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Abstract

Forty-six bacterial cultures, including one culture collection strain, thirty from the rhizosphere of *Alyssum murale* and fifteen from Ni-rich soil, were tested for their ability to tolerate arsenate, cadmium, chromium, zinc, mercury, lead, cobalt, copper, and nickel in their growth medium. The resistance patterns, expressed as minimum inhibitory concentrations, for all cultures to the nine different metal ions were surveyed by using the agar dilution method. A large number of the cultures were resistant to Ni (100%), Pb (100%), Zn (100%), Cu (98%), and Co (93%). However, 82, 71, 58 and 47% were sensitive to As, Hg, Cd and Cr(VI), respectively. All cultures had multiple metal-resistant, with heptametal resistance as the major pattern (28.8%). Five of the cultures (about of 11.2% of the total), specifically *Arthrobacter rhombi* AY509239, *Clavibacter xyli* AY509235, *Microbacterium arabinogalactanolyticum* AY509226, *Rhizobium mongolense* AY509209 and *Variovorax paradoxus* AY512828 were tolerant to nine different metals. The polymerase chain reaction in combination with DNA sequence analysis was used to investigate the genetic mechanism responsible for the metal resistance in some of these gram-positive and gram-negative bacteria that were, highly resistant to Hg, Zn, Cr and Ni. The *czc*, *chr*, *ncc* and *mer* genes that are responsible for resistance to Zn, Cr, Ni and Hg, respectively, were shown to be present in these bacteria by using PCR. In the case of, *M. arabinogalactanolyticum* AY509226 these genes were shown to have high homology to the *czc*D, *chr*B, *ncc*A, and *mer* genes of *Ralstonia metallidurans* CH34. Therefore, Hg, Zn, Cr and Ni resistance genes are widely distributed in both gram-positive and gram-negative isolates obtained from *A. murale* rhizosphere and Ni-rich soils.

Keywords: Heavy metals; *Alyssum murale*; Serpentine soil; Bacteria; PCR; Metal resistance genes

1. Introduction

Serpentine (ultramafic) outcrops are distributed all over the world and, for their natural geological origin, are characterized by high levels of cobalt, chromium, and especially nickel (Brooks, 1987). A number of plant species endemic to metalliferous soils are capable of accumulating exceptional concentrations of metals, such as nickel, zinc and cobalt, to levels that greatly exceed those normally considered to be phytotoxic (Baker and Brooks, 1989). These plants are known as metal 'hyperaccumulators' (Brooks et al., 1977). *Alyssum murale* is the best-known among hyperaccumulators and has the ability to colonize serpentine soils and accumulate nickel in excess of 2% (W/W) of shoot dry-matter (Reeves and Baker, 2000).

Bacteria present in serpentine soils and their interaction with hyperaccumulating plants have been the focus of several investigators (Schlegel et al., 1991; Mengoni et al.,

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2001; Abou-Shanab et al., 2003a,b). Bacterial communities in serpentine soil were reported to tolerate spiking of metals, such as nickel and zinc, more than those of unpolluted soils. Also, evidence was presented that the soil near hyperaccumulating plants, such as *Sebertia acuminata*, *Thlaspi caerulescens*, *Alyssum bertolonii* and *A. murale* has an increased proportion of bacteria with metal-resistance.

Low concentrations of certain transition metals such as cobalt, copper, nickel and zinc are essential for many cellular processes of bacteria. However, higher concentrations of these metals often are cytotoxic. Other heavy metals, including lead, cadmium, mercury, silver and chromium have no known beneficial effects to bacterial cells and are toxic even at low concentrations (Nies, 2004). Microbial survival in polluted soils depends on intrinsic biochemical and structural properties, physiological, and/ or genetic adaptation including morphological, changes of cells, as well as environmental modifications of metal speciation (Wuertz and Mergeay, 1997). Microbes apply various types of resistance mechanisms in response to heavy metals (Nies, 2003). These mechanisms may be encoded by chromosomal genes, but more usually loci conferring resistance are located on plasmids (Cervantes and Gutierrez-Corona, 1994; Wuertz and Mergeay, 1997).

Investigations of adaptive responses commonly involve studying phenotypic changes. However, a more basic understanding of adaptation is possible if the molecular mechanisms of resistance is also characterized. Approaches that can be used include the use of molecular techniques such as the polymerase chain reaction (PCR), DNA–DNA hybridization and an analysis of restriction fragment length polymorphism (RFLP) (Barkay et al., 1985; Diels and Mergeay, 1990; Rochelle et al., 1991; Nakamura and Silver, 1994). These techniques are, in general, more sensitive and rapid than some of the traditional methods. A significant advantage is that these approaches can be aimed precisely at a particular genetic determinant and thereby may provide a useful means of investigating bacterial responses to environmental stress and reveal the molecular mechanisms of adaptation.

This paper describes, the heavy metal resistance properties of forty-five bacteria that were isolated either from Ni-rich soils collected in Oregon or the rhizosphere of *A. murale* grown in the same soils. The study also included an analysis of several genes implicated in metal resistance that were present in some of these isolates.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Isolation of the bacterial cultures used in this work, is described in Abou-Shanab et al. (2003b) and cultures used in this study are listed in Table 1. The cultures included isolates from the rhizosphere of *A. murale* grown in serpentine Ni-rich soils as well as those originating from unplanted serpentine soils. Tris-buffered mineral salts medium (Mergeay et al., 1985) containing 0.2% (w/v) sodium gluconate as a carbon source was used for testing resistance to heavy metals and for growing bacterial cultures at 30 °C. For plating, growth media were solidified with 15 g of agar per liter.

2.2. Estimation of bacterial tolerance to metals

Analytical grades of metal salts ($CdCl_2 \cdot 6H_2O$; $CoCl_2 \cdot 6H_2O$; $CuCl_2 \cdot 2H_2O$; $K_2Cr_2O_7$; $NiCl_2 \cdot 6H_2O$; $Hg(CH_3COO)_2$; $Pb(NO_3)_2$; ZnSO4 and $NaAsO_2$) were used to prepare 0.125 M stock solutions. Each stock solution was filter-sterilized and added to tris-buffered low-phosphate agar (TBLPA) medium (Mergeay et al., 1985) to final concentrations of 0.01; 0.02; 0.05; 0.1; 0.2; 0.5; 1.0; 2.0; 5.0; 10; 20 and 40 mM of each metal for determinations of the minimum inhibitory concentrations (MICs) of the metal ions for each isolate. The agar plate method was used since this is an accepted approach that has been used in many reported studies (Mergeay et al., 1985; Siddiqui et al., 1989; Schlegel et al., 1991; Liesegang et al., 1993; Taghavi et al., 1997). Cultures were grown overnight in Tris minimal broth and then 10 μl of each of the cultures were spotted onto metal salt-containing TBLPA plates. In this way 15 cultures per plate could be conveniently tested. Duplicate plates were prepared for each metal concentration and then they were incubated at 30 °C. Each plate was checked for growth at 2 days intervals for at least one week and positives were recorded by the appearance of colonies on the plate surface. TBLPA agar plates without heavy metals were used as controls. The lowest concentration that prevented growth was considered the MIC. For the purpose of defining metal resistance, those isolates that grew in the presence of 10 mM As and 1 mM each of Cd, Co, Cu, Ni, Pb, Zn and Cr, and 0.1 mM Hg were considered to be resistant (Nieto et al., 1987).

2.3. Isolation of total genomic DNA

Total genomic DNA of several metal resistant isolates was extracted from 5 ml cultures grown overnight in R2A broth medium (Reasoner and Geldreich, 1985) using a small-scale tissue and blood DNA extraction kit (Qiagen Inc., Chatsworth, CA, USA) according to the manufacturer's instructions and protocols.

2.4. PCR analysis using czc, ncc, chr and mer primers

Oligonucleotide sequences used as primers for the partial amplification of the *czc*D, *chr*B, *mer*A and *ncc* loci are given in Table 2. The ncc operon was amplified as a 1141-bp fragment that spanned the *nccA* and *nccN* genes. Templates for PCR amplification included the total genomic DNA from a selection of highly metal resistant gram-positive and gram-negative bacteria and also included *Alcaligenes eutrophus* CH34 as a positive control. The reaction mixtures were 100 μl final volumes with

Table 1
Bacterial strains used in this study

Bacterial strain	Accession no.	Origin	Reference or source
Alcaligenes eutrophus	X58441	ATCC[a]	# 43123
Acidovorax avenae	AY512827	R[b]	Abou-Shanab et al. (2003b)
Acidovorax delafieldii	AY512826	R	as above
Arthrobacter ramosus	AY509238	R	as above
Arthrobacter rhombi	AY509239	R	as above
Bacillus flexus	AY509229	S[c]	as above
Bacillus niacini	AY509227	S	as above
Bacillus niacini	AY509228	S	as above
Bacillus psychrosaccharolyticus	AY509230	S	as above
Burkholderia cepacia	AY512825	R	as above
Caulobacter crescentus	AY512823	R	as above
Clavibacter xyli	AY509235	R	as above
Clavibacter xyli	AY509236	R	as above
Clavibacter xyli	AY509237	R	as above
Massilia timonae	AY512824	R	as above
Mesorhizobium loti	AY509218	S	as above
Microbacterium arabinogalactanolyticum	AY509224	R	as above
Microbacterium arabinogalactanolyticum	AY509225	R	as above
Microbacterium arabinogalactanolyticum	AY509226	R	as above
Microbacterium liquefaciens	AY509220	R	as above
Microbacterium oxydans	AY509219	R	as above
Microbacterium oxydans	AY509221	S	as above
Microbacterium oxydans	AY509222	R	as above
Microbacterium oxydans	AY509223	R	as above
Nocardioides simplex	AY509240	S	as above
Paenibacillus amylolyticus	AY509232	S	as above
Paenibacillus amylolyticus	AY509233	S	as above
Paenibacillus amylolyticus	AY509234	S	as above
Paenibacillus lautus	AY509231	S	as above
Phyllobacterium myrsinacearum	AY512821	R	as above
Pseudomonas riboflavina	AY512822	R	as above
Rhizobium etli	AY509210	R	as above
Rhizobium etli	AY460185	R	as above
Rhizobium galegae	AY509213	R	as above
Rhizobium galegae	AY509214	R	as above
Rhizobium galegae	AY509216	R	as above
Rhizobium galegae	AY509215	R	as above
Rhizobium gallicum	AY509211	R	as above
Rhizobium mongolense	AY509212	R	as above
Rhizobium mongolense	AY509209	S	as above
Sinorhizobium fredii	AY509217	S	as above
Sphingomonas alaskensis	AY509242	S	as above
Sphingomonas asaccharolytica	AY509241	R	as above
Sphingomonas macrogoltabidus	AY509243	R	as above
Stenotrophomonas minatitlanensis	AY512829	S	as above
Variovorax paradoxus	AY512828	R	as above

[a] ATCC – American Type Culture Collection.
[b] *Alyssum murale* rhizosphere soil.
[c] Unplanted Ni rich-soil.

Epicentre Biotechnologies Master Amp Taq DNA polymerase and Epicentre failsafe PCR 2x premix buffers D, E, F and G for amplification of *chr*B, *ncc*, *czc*D and *mer*A, respectively. For PCR a PTC-225 Peltier thermal cycler DNA engine tetrad was used with a 35 cycle program. An initial denaturation at 95 °C for 5 min was followed by annealing at 57 °C for 1 min, extension at 72 °C for 1.5 min and denaturation at 94 °C for 30 s. This was followed by a final extension of 3 min at 72 °C. Product formation was confirmed by 1.5% (w/v) agarose gel electrophoresis and visualization with ultraviolet illumination after staining with 0.5 μg ml^{-1} ethidium bromide.

2.5. Sequence analysis

PCR products were purified using QIAquick Spin columns (Qiagen Inc., Chatsworth, CA). An Applied Biosystem 3100 Genetic analyzer DNA sequencer in combination with a Dye Deoxy Terminator Cycle Sequencing Kit (Perkin–Elmer, Foster City, CA) were used for sequencing

Table 2
Oligonucleotide primers used for PCR amplification

Resistance determinant amplified	Sequence 5′–3′	Orientation	Exact length of amplified region (bp)	References
*mer*A	GAGATCTAAAGCACGCTAAGGC	Forward	1011	Misra et al. (1984)
	GGAATCTTGACTGTGATCGGG	Reverse		
*chr*B	GTCGTTAGCTTGCCAACATC	Forward	450	Nies et al. (1990)
	CGG AAAGCAAGATGTCGATCG	Reverse		
*czc*D	TTTAGATCTTTTACCACCATGGGCGCAGGTCACTCACACGACC	Forward	1000	Nies et al. (1989)
	TTTCAGCTGAACATCATACCCTAGTTTCCTCTGCAGCAAGCGACTTC	Reverse		
*ncc*A	ACGCCGGACATCACGAACAAG	Forward	1141	This study
	CCAGCGCACCGAGACTCATCA	Reverse		

the purified PCR products as described previously (van Berkum et al., 1996). The basic local alignment search tool (BLAST) searches (Altschul et al., 1997) of GenBank were done to obtain entries with similar sequences. Sequences similar to *czc*D, *mer*A, *ncc*A and *chr*A were downloaded and were aligned with our datasets using the PILEUP program in the Wisconsin package of the Genetics Computer group (Madison, WI, USA). Aligned sequences were checked manually and were edited with Genedoc (Nicholas and Nicholas, 1997).

3. Results and discussion

3.1. Response of bacterial isolates to heavy metals

The percentage of the isolates that were susceptible when challenged with various concentrations of the nine heavy metal ions are shown in Table 3. The frequencies of resistance for all isolates to each metal ion tested were as follows: As, 18%; Cd, 42%; Co, 93%; Cr (VI), 53%; Cu, 98%; Hg, 29%; Ni, 100%; Pb, 100%; and Zn, 100%. Mercury was the most toxic inhibiting 7% of the isolates at 0.01 mM (Table 3). The order of toxicity of the metals was found to be Hg > Cd > Co > Cr > Cu > As > Zn > Pb > Ni. In general, the toxic effect of these metals increased with increasing concentration. A large proportion of the isolates were resistant to Ni (100%) and Co (93%).

From comparisons of the results across the metals, it was evident that 55.5%, 31.1%, 28.9%, 20%, 13.3%, 11.1%, 11.1%, 6.6%, and 6.6% of the isolates were tolerant to Ni, Cr, Zn, Cd, Hg, As, Pb, Cu, and Co ions with MICs of 15, 5, 10, 5, 0.5, 20, 15, 15 and 10 mM, respectively (Table 4). All the cultures showed some tolerance to heavy metals with a large proportion even tolerating 20, 10, and 10 mM Ni, Pb, and Zn, respectively.

The high levels of resistance and the widespread tolerance that was found among the isolates is probably attributed to the high metal contents (4, 390 mg Ni kg^{-1} and 330 mg Co kg^{-1}) of Oregon soils (Abou-Shanab et al., 2003b). In this soil the bacteria would have been exposed to the heavy metals since they are in forms that are available either in solution or adsorbed on soil colloids (Giller et al., 1998). Metal exposure probably led to selection of tolerance among members of the bacterial populations, which predominantly are gram-positive and gram-negative (Wuertz and Mergeay, 1997; Kozdroj and van Elsas, 2001; Abou-Shanab et al., 2003b).

All the isolates were tolerant to multiple metal ions. However, the patterns of tolerance among the 45 cultures varied (Table 5). The incidence of bacteria with tolerance to various hepta and hexa metal ions was significant (about 46.4% of the total). Five of the isolates; *Arthrobacter rhombi* AY509239, *Clavibacter xyli* AY509235, *Microbacterium arabinogalactanolyticum* AY509226, *Rhizobium mongolense* AY509209 and *Variovorax paradoxus* AY512828 were tolerant to nine different metals. Similarly, Nieto et al. (1987) reported that *Halobacterium mediterranei* ATCC 33500 was tolerant to eight different metals.

Table 3
Susceptibility of 45 soil bacterial strains to 9 metal ions

Metal ion	Cumulative % of strains susceptible to the following metal ion concentration (mM)											
	0.005	0.01	0.05	0.1	0.5	1	2.5	5	10	15	20	40
As	0	0	0	0	2	20	58	71	82	82	89	100
Cd	0	0	0	11	47	58	62	80	100	100	100	100
Co	0	0	0	0	4	7	53	73	100	100	100	100
Cr	0	0	0	0	33	47	53	69	100	100	100	100
Cu	0	0	0	0	0	2	42	51	67	93	100	100
Hg	0	7	36	71	87	100	100	100	100	100	100	100
Ni	0	0	0	0	0	0	0	7	20	44	100	100
Pb	0	0	0	0	0	0	0	2	33	87	100	100
Zn	0	0	0	0	0	0	0	27	71	100	100	100

Table 4
MICs of 9 metal ions tested against bacterial strains isolated from rhizosphere of *Alyssum murale* and unplanted Ni-rich serpentine soil

Microorganism	MIC (mM)								
	As	Cd	Co	Cr	Cu	Hg	Ni	Pb	Zn
Alcaligenes eutrophus X58441	5	10	15	2.5	5	1	10	15	20
Acidovorax avenae AY512827	2.5	2.5	5	0.5	2.5	0.05	10	10	10
Acidovorax delafieldii AY512826	0.5	0.1	1	0.1	1	0.01	5	5	5
Arthrobacter ramosus AY509238	1	2.5	2.5	5	2.5	0.05	15	10	5
Arthrobacter rhombi AY509239	20	5	5	5	10	0.5	15	10	10
Bacillus flexus AY509229	0.5	0.05	1	0.1	1	0.01	5	10	0.5
Bacillus niacini AY509227	2.5	0.1	2.5	5	15	0.1	15	5	5
Bacillus niacini AY509228	1	0.05	1	0.1	1	0.01	10	5	5
Bacillus psychrosaccharolyticus AY509230	1	5	2.5	1	5	0.01	10	10	5
Burkholderia cepacia AY512825	2.5	2.5	10	5	1	0.05	15	15	5
Caulobacter crescentus AY512823	0.5	0.1	0.5	0.1	1	0.005	10	5	0.5
Clavibacter xyli AY509235	5	5	2.5	2.5	5	0.1	15	5	10
Clavibacter xyli AY509236	1	5	1	2.5	1	0.05	15	10	10
Clavibacter xyli AY509237	1	5	1	2.5	10	0.01	15	10	10
Massilia timonae AY512824	20	0.1	5	2.5	1	0.01	15	5	10
Mesorhizobium loti AY509218	0.1	0.1	0.1	0.1	0.5	0.005	5	5	0.5
Microbacterium arabinogalactanolyticum AY509224	1	2.5	2.5	5	10	0.05	15	10	10
Microbacterium arabinogalactanolyticum AY509225	20	0.5	5	5	2.5	0.05	15	5	10
Microbacterium arabinogalactanolyticum AY509226	20	2.5	5	5	10	0.1	15	15	5
Microbacterium liquefaciens AY509220	5	0.5	0.1	2.5	2.5	0.05	15	10	5
Microbacterium oxydans AY509219	1	1	1	5	5	0.05	15	10	10
Microbacterium oxydans AY509221	1	2.5	2.5	1	5	0.1	5	10	5
Microbacterium oxydans AY509222	15	5	5	5	10	0.05	15	15	10
Microbacterium oxydans AY509223	1	1	1	5	15	0.05	15	10	5
Nocardioides simplex AY509240	0.5	0.05	1	0.1	15	0.01	5	10	2.5
Paenibacillus amylolyticus AY509232	1	0.1	1	2.5	1	0.05	2.5	10	2.5
Paenibacillus amylolyticus AY509233	5	0.1	1	1	1	0.05	2.5	10	2.5
Paenibacillus amylolyticus AY509234	15	0.1	1	0.1	1	0.05	10	5	5
Paenibacillus lautus AY509231	5	0.1	1	0.1	5	0.05	5	5	5
Phyllobacterium myrsinacearum AY512821	2.5	0.5	1	0.5	10	0.05	15	10	10
Pseudomonas riboflavina AY512822	1	5	5	0.5	10	0.1	10	10	5
Rhizobium etli AY509210	0.5	0.1	1	0.5	1	0.005	15	15	0.5
Rhizobium etli AY460185	2.5	5	10	5	10	0.1	15	15	10
Rhizobium galegae AY509213	0.5	0.1	1	0.5	1	0.01	15	2.5	2.5
Rhizobium galegae AY509214	1	2.5	2.5	2.5	10	0.05	15	10	5
Rhizobium galegae AY509216	1	0.1	10	5	10	0.01	15	10	0.5
Rhizobium galegae AY509215	5	0.1	1	0.1	10	0.5	15	10	2.5
Rhizobium gallicum AY509211	1	0.1	1	0.1	1	0.01	10	5	10
Rhizobium mongolense AY509212	1	0.1	2.5	0.1	1	0.01	10	10	5
Rhizobium mongolense AY509209	15	2.5	2.5	5	1	0.5	10	10	5
Sinorhizobium fredii AY509217	0.5	0.1	1	0.1	1	0.01	2.5	5	0.5
Sphingomonas alaskensis AY509242	1	0.1	1	0.1	5	0.5	15	5	5
Sphingomonas asaccharolytica AY509241	0.5	0.1	1	0.1	1	0.01	10	5	0.5
Sphingomonas macrogoltabidus AY509243	1	0.5	5	0.5	5	0.5	15	10	5
Stenotrophomonas minatitlanensis AY512829	1	0.5	1	0.1	1	0.5	10	10	5
Variovorax paradoxus AY512828	20	5	5	5	10	0.1	15	10	5

3.2. Amplification of czc, chr, mer and ncc genes

The Gram negative isolates, *Rhizobium etli* AY460185, *Acidovorax avenae* AY512827, *Massilia timonae* AY512824, *Rhizobium gallicum* AY509211 and *V. paradoxus* AY512828 and the gram-positive isolates, *M. arabinogalactanolyticum* AY509224, *M. oxydans* AY509219, *Clavibacter xyli* AY509236, *M. arabinogalactanolyticum* AY509225, *C. xyli* AY509237, and *Arthrobacter rhombi* AY509239 were the most resistant to zinc. The MICs for zinc in these isolates were approximately half that of *Alcaligenes eutrophus* CH34, which is known to be zinc resistant (Table 4). Presumptive evidence for the presence of the locus czc in the genomes of all 11 isolates was obtained by using the primer pair *czc*D1 and *czc*D2. PCR products generated from the DNA of the 11 isolates were identical in size (about 1 kb) to that obtained with the positive control (*A. eutrophus*), which indicated that zinc resistance probably was mediated by the *czc* operon (Fig. 1).

Evidence that chromate resistance in the gram-positive isolates *M. arabinogalactanolyticum* AY509224, *M. oxydans* AY509223, *M. arabinogalactanolyticum* AY509225, *A. rhombi* AY509239, *A. ramosus* AY509238, *Bacillus niacini* AY509227, and the gram-negative isolates *Burkholderia*

Table 5
Patterns of tolerance of 9 heavy metal ions in 45 soil bacterial strains

No. of different tolerance	Types of tolerance	No. (%) of strains
9	Cr, Cu, Ni, Cd, Zn, Pb, Co, Hg, As	5 (11.2)
8	Cr, Cu, Ni, Cd, Zn, Pb, Co, Hg	2 (4.4)
	Cr, Cu, Ni, Cd, Zn, Pb, Co, As	1 (2.2)
7	Cr, Cu, Ni, Zn, Pb, Co, Cd,	9 (20)
	Cr, Cu, Ni, Zn, Pb, Co, As	2 (4.4)
	Cr, Cu, Ni, Zn, Pb, Co, Hg	1 (2.2)
	Cd, Cu, Ni, Zn, Pb, Co, Hg	1 (2.2)
6	Hg, Cu, Ni, Zn, Pb, Co	4 (8.8)
	As, Cu, Ni, Zn, Pb, Co	1 (2.2)
	Cr, Cu, Ni, Zn, Pb, Co	2 (4.4)
	Cd, Cu, Ni, Zn, Pb, Co	1 (2.2)
5	Zn, Co, Cu, Ni, Pb	8 (17.7)
	Cr, Co, Cu, Ni, Pb	1 (2.2)
	Cr, Zn, Cu, Ni, Pb	1 (2.2)
4	Co, Cu, Ni, Pb	4 (8.8)
3	Cu, Ni, Pb	1 (2.2)
2	Ni, Pb	1 (2.2)

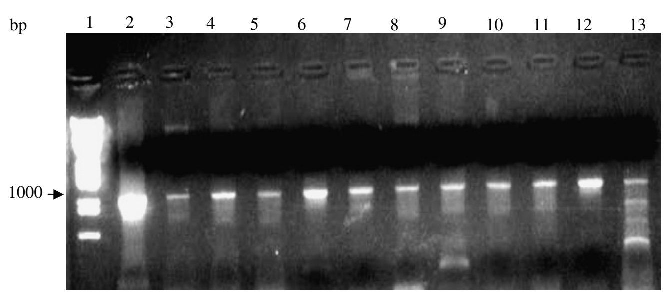

Fig. 1. Agarose gel electrophoresis of *czc*D PCR products. Lanes: 1, DNA size marker (Lambda DNA digested with *Hin*DIII and *Eco*R1); 2, *Alcaligenes eutrophus* Ch34 (ATTC#43123; 3, *Microbacterium arabinogalactanolyticum* AY509224; 4, *M. oxydans* AY509219; 5, *Clavibacter xyli* AY509236; 6, *Rhizobium etli* AY460185; 7, *Acidovorax avenae* AY512827; 8, *Massilia timonae* AY512824; 9, *M. arabinogalactanolyticum* AY509225; 10, *C. xyli* AY509237; 11, *Arthrobacter rhombi* AY509239; 12, *R. gallicum* AY509211 and 13, *Variovorax paradoxus* AY512828.

cepacia AY512825, *Rhizobium etli* AY460185, *R. galegae* AY509216, *R. mongolense* AY509209 and *V. paradoxus* AY512828 was mediated by the chr operon was obtained by PCR using the *chr*B1–*chr*B2 primer pair. PCR with this primer pair yielded the expected ~450 bp products, which was similar to that obtained with the positive control *A. eutrophus* CH34 (Fig. 2). These isolates were more resistant to Cr(VI) than *A. eutrophus* CH34 with MICs that were approximately two-fold that of the control (Table 4).

Presumptive evidence for the presence of the *mer* and *ncc* loci that confer resistance to mercury and nickel, respectively, was also obtained by PCR. The primer pair *mer*1–*mer*2 yielded an expected ~1011 bp. PCR product in *C. xyli* AY509235, *R. etli* AY460185, *A. rhombi* AY509239, *M. arabinogalactanolyticum* AY509226, *R. mongolense* AY509209 and *Sphingomonas alaskensis* AY509242 similar to the positive control *A. eutrophus* (Fig. 3). However, no or faint products were obtained with (*M. arabinogalactanolyticum* AY509224, *Sphingomonas macrogoltabidus* AY509243, *R. galegae* AY509215, *M. oxydans* AY509221 and *Stenotrophomonas minatitlanensis* AY512829). In the case of nickel resistance, the primer pair *ncc* upper and ncc lower yielded the expected ~1141 bp product with the gram-negative *R. gallicum* AY509211 and in the gram-positive *M. arabinogalactanolyticum* AY509224 similar to *A. eutrophus* CH34, in (Fig. 4). However, no PCR product was generated with the other twelve isolates that were resistant to 10–15 mM nickel (data not shown).

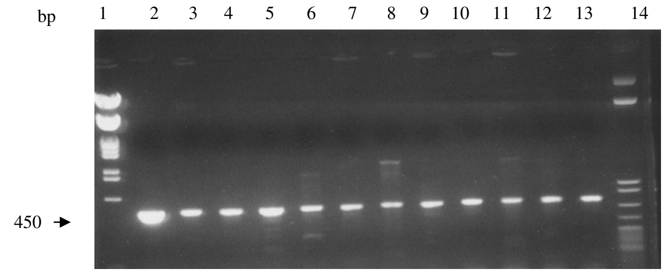

Fig. 2. Agarose gel electrophoresis of *chrB* PCR products. Lanes: 1, DNA size marker (Lambda DNA digested with *Hin*DIII and *Eco*R1); 2, *Alcaligenes eutrophus* Ch34 (ATTC#43123; 3, *Microbacterium arabinogalactanolyticum* AY509224; 4, *M. oxydans* AY509223; 5, *M. arabinogalactanolyticum* AY509225; 6, *Burkholderia cepacia* AY512825; 7, *Rhizobium etli* AY460185; 8, *R. galegae* AY509216; 9, *Arthrobacter rhombi* AY509239; 10, *A. ramosus* AY509238; 11, *Variovorax paradoxus* AY512828; 12, *R. mongolense* AY509209; 13, *Bacillus niacini* AY509227 and 14, pBR322 DNA/*AIu*I Marker.

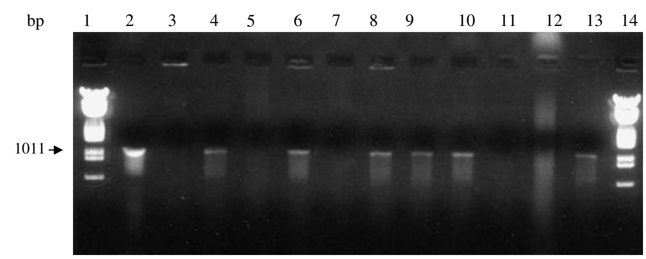

Fig. 3. Agarose gel electrophoresis of *mer* PCR products. Lanes: 1, DNA size marker (Lambda DNA digested with *HinD*III and *Eco*R1); 2, *Alcaligenes eutrophus* Ch34 (ATTC#43123); 3, *Microbacterium arabinogalactanolyticum* AY509224; 4, *Clavibacter xyli* AY509235; 5, *Sphingomonas macrogoltabidus* AY509243; 6, *Rhizobium etli* AY460185; 7, *R. galegae* AY509215; 8, *Arthrobacter rhombi* AY509239; 9, *M. arabinogalactanolyticum* AY509226; 10, *R. mongolense* AY509209; 11, *M. oxydans* AY509221; 12, *Stenotrophomonas minatitlanensis* AY512829; 13, *Sphingomonas alaskensis* AY509242 and 14, DNA size marker (Lambda DNA digested with *Hin*DIII and *Eco*R1).

3.3. Sequence analysis of czcD, chrB, mer and nccA genes

The gram-positive culture *M. arabinogalactanolyticum* AY509226 that is resistant to Cr(VI), Hg^{2+}, Ni^{2+} and

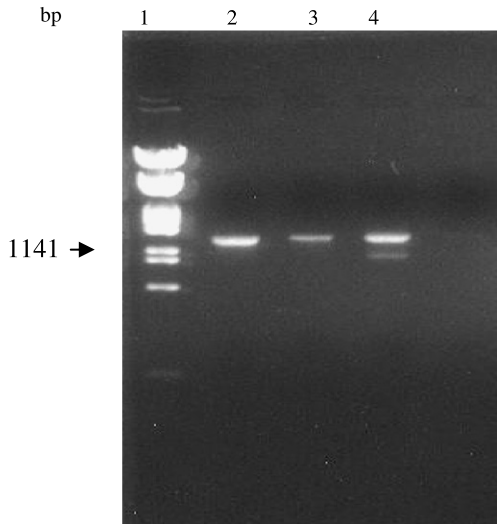

Fig. 4. Agarose gel electrophoresis of *ncc* PCR products. Lanes: 1, DNA size marker (Lambda DNA digested with *Hin*DIII and EcoR1); 2, *Alcaligenes eutrophus* Ch34 ATTC#43123; 3, *Microbacterium arabinogalactanolyticum* AY509224 and 4, *Rhizobium gallicum* AY509211.

Zn^{2+} (Table 4) was chosen for sequence analysis of the *czc*D, *chr*B, *ncc*A and *mer* genes. The partial nucleotide sequences obtained were 100%, 100% and 99% similar with *czc*D, *chr*B and *ncc*A loci of the gram-negative *A. eutrophus* CH34 (Nies et al., 1989; Liesegang et al., 1993; Diels et al., 1995). The partial nucleotide sequences obtained for *mer*P, *mer*T, and *mer*R, also were 100% similar with the loci in *A. eutrophus* CH34 that are located on plasmid pMOL30 (Diels et al., 1995).

Metal resistance has been reported for a number of gram-negative bacteria belonging to the *Ralstonia* lineage of the ß-Proteobacteria (Schmidt and Schlegel, 1994). *Ralstonia eutropha* strain CH34 (formerly *A. eutrophus* strain CH34 (Taghavi et al., 1997)) possesses at least seven determinants encoding resistance to toxic heavy metals. These loci are located either within the bacterial chromosome or on one of the two plasmids pMOL28 or pMOL30 (Mergeay et al., 1985; Siddiqui et al., 1989; Liesegang et al., 1993). The replicon pMOL30 harbors the *czc* operon, which encodes resistance to Cd^{2+}, Zn^{2+} and Co^{2+}, the *cop* locus (Cu^{2+} resistance), the *pbr* locus (Pb^{2+} resistance), the thallium resistance locus, *tll*B, the gene for Hg^{2+} resistance on transposon Tn4380, the *cnr* operon (Co^{2+}, Ni^{2+}, and Zn^{2+} resistance; ZinB phenotype) and the *chr* operon for CrO_4^{2-} resistance (Collard et al., 1993; Mergeay et al., 2003). Because these metal resistance determinants are commonly located on plasmids or on transposons, the suggestion has been made that these genes may be spread to divergent bacteria by horizontal transfer (Barkay et al., 1985; Bogdanova et al., 1988). The detection of these genes within the genome of *M. arabinogalactanolyticum* AY509226 in this work would provide evidence that these genes are shared both within as well as across the gram-positive and gram-negative bacterial communities.

4. Conclusions

Forty-five gram-positive and gram-negative soil bacteria originating from the *A. murale* rhizosphere and unplanted Ni-rich soil exhibited resistance to a range of metal ions that included arsenate, lead, cadmium, mercury, nickel, cobalt, copper, chromium and zinc. From PCR and DNA sequence analysis evidence was provided that the loci conferring resistance to these metals are present within both the gram-positive and gram-negative bacterial communities. Horizontal gene transfer across bacterial communities and the subsequent selection for metal resistance in the serpentine soil is the most likely mechanism to explain why a gram-positive isolate possessed metal resistance genes that were identical with the previously reported gene sequences in *R. metallidurans* CH34. It is suggested that the isolate *M. arabinogalactanolyticum* AY509226 is a bacterial model for eco-toxicological studies because of a relatively high MIC for metals and a large spectrum of metal resistance.

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